Sec translocase in action

Komarudin, Amalina Ghaisani

CHAPTER 1

SecA Mediated Protein Translocation Through SecYEG Channel

Amalina Ghaisani Komarudin and Arnold J.M. Driessen

submitted

ABSTRACT

In bacteria, proteins are synthesized at ribosomes localized in the cytosol. Proteins that reside outside the cytoplasmic membrane need to be targeted to the Sec translocase, the major system for the translocation of proteins across the cytoplasmic membrane. The Sec translocase consists of a hetero-trimeric protein conducting channel SecYEG, the peripheral ATP-dependent motor protein SecA, and the SecDF complex that accelerates translocation at the expense of the proton motive force. Together these proteins form the holo-translocon. Here, we provide an overview of the current knowledge on the function and structure of the Sec translocase, with an emphasis on the minimal functional unit of SecA and SecYEG.

INTRODUCTION

Protein transport is an orchestrated process common to all domains of life [1]. Proteins that need to function outside the cytosol are translocated across the lipid membrane barrier. This transport process is mediated by the translocon domain of the Sec translocase: SecYEG in bacteria [2], SecYEβ in archaea [3] and Sec61αβγ in the membrane of the endoplasmic reticulum of eukaryotic cells [4]. The translocon forms a protein conducting channel in the membrane, and conducts the membrane passage of unfolded signal peptide bearing preproteins and are subsequently released at the *trans* side of the membrane where they fold into their native structure once the signal peptide has been removed [5]. In addition, the Sec translocase mediates the co-translational insertion of nascent membrane proteins into the cytoplasmic membrane. In general, two modes of activity can be distinguished: translocation of preproteins after they are fully synthesized (post translational pathway) and insertion of membrane proteins while they are still being translated by the ribosomes (co-translational pathway) (Fig.1).

In the post-translational pathway, preproteins are synthesized with a cleavable amino-terminal signal sequence. These proteins are bound by the molecular chaperone SecB which maintains them in a translocation-competent conformation [6] corresponding to a state lacking stable tertiary structure. SecB targets preproteins to the SecYEG-bound SecA [7,8]. SecA is a motor protein and its ATPase activity provides the energy for the translocation process [9]. ATP-induced conformational changes of SecA drive the stepwise translocation of preproteins through the translocon [2]. During translocation, the signal sequence is cleaved off by a membrane bound signal peptidase to yield the mature protein at the *trans* side of the membrane [10]. SecYEG also associates with accessory Sec components, most notably the SecDFyajC complex [11]. Large segments of the preprotein may be translocated in the presence of the PMF once SecA has released the preprotein. This process involves the SecDF membrane protein complex [12].

In the co-translational pathway, the nascent proteins are guided to the translocon by signal recognition particle (SRP). As soon as a nascent membrane protein emerges from the ribosome, its first transmembrane domain (or a highly hydrophobic signal sequence) is recognized by SRP to form a ternary nascent chain-SRP-ribosome (RNC) complex. SRP targets this complex to the SRP receptor, FtsY at the membrane where SRP and FtsY form a heterodimer. Subsequently GTP binding reactions to the SRP-FtsY heterodimer result in the release of the nascent chain from SRP and a transfer to the translocon. Eukaryotes may use this pathway both for protein secretion and membrane

protein insertion whereas in bacteria it is mostly used for membrane protein insertion [13]. This review focusses on protein translocation.

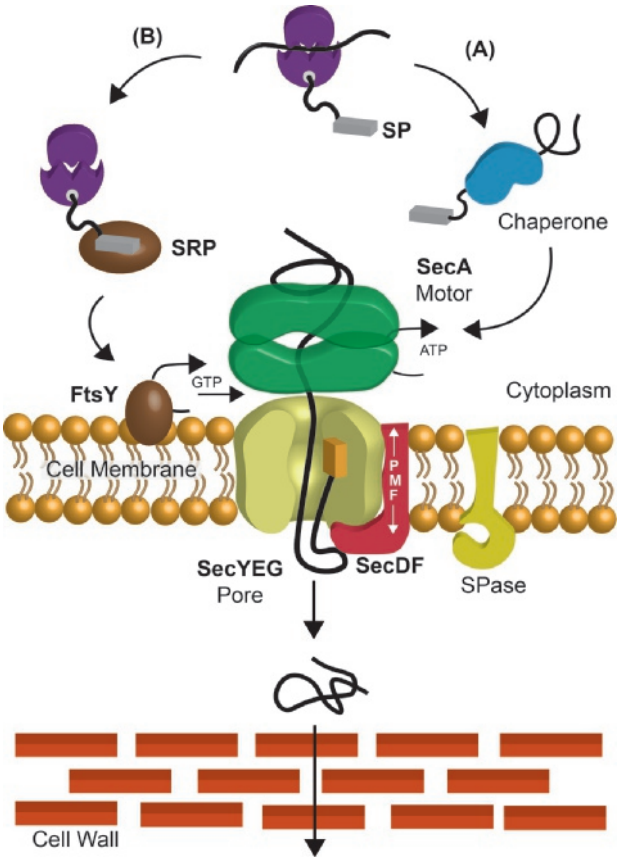

Fig. 1 The Sec pathway. (A) Post translational pathway: after complete synthesis of the preproteins at the ribosome, the unfolded preprotein is recognized by the molecular chaperone SecB (blue) and targeted to SecA (green). SecA guides the preprotein through the SecYEG pore (lime) employing the energy from ATP binding and hydrolysis. The signal peptide is cleaved by the signal peptidase (SP, yellow). SecDF (pink) pull the preprotein at the expense of the PMF. (B) Co-translational pathway: once a hydrophobic transmembrane domain of a nascent membrane protein emerges from the ribosomes, signal recognition particle (SRP) binds to the ribosome:nascent chain and guides the complex to the SR receptor FtsY (brown) at the cytoplasmic membrane where the ribosome nascent chain is released from SRP and transferred to the SecYEG channel upon the binding of GTP to the SRP:FtsY heterodimer. Next, membrane protein synthesis at the ribosome is directly linked to the SecYEG dependent inserts into the membrane.

SECYEG, THE PROTEIN CONDUCTING CHANNEL

SecYEG forms the core of the Sec translocase. This complex has been crystalized in different states, and structures have been obtained from both bacterial and archaeal translocons. Recent studies suggest that these structures that can be categorized into: 1) a resting or closed state (PDB entry 1RH5) [14], 2) a pre-opened state primed by SecA (PDB entry 3DIN) [15], and 3) an active state engaged or in complex with a signal peptide substrate [16] (PDB entry 5EUL) (Fig. 2).

SecYEG structure

Based on the X-ray structure of SecYEβ from *Methanocaldococcus jannaschii*, and all other structures, SecY consists of two halves formed by transmembrane segments (TMS) 1-5 and 6-10 [14]. The two halves are connected by a loop of TMS 5/6 resulting in a clamshell-like structure of the translocon. Molecular dynamics simulations suggest that the short loop at the hinge (HL-1) allows clamshell opening [17]. SecY is shaped like an hourglass with a funnel-like entrance and a subcentral constriction. During translocation, clamshell opening results in a widening of the subcentral constriction allowing the preprotein to insert into the channel. The front of SecY between TMS 2 and 7 forms a lateral gate which opens towards the lipid bilayer [14]. At the periplasmic face of the membrane, the exit site of the funnel shaped pore is closed by an α-helical plug (TMS2a) that folds back into the channel [18,19]. The *M. jannaschii* SecYEβ structure is consider to be in a resting/closed state with a sealed pore where six hydrophobic residues closed the central constriction ring and with the plug closing the exit funnel [14].

SecE surrounds SecY at the back of the complex and embraces the SecY clamshell structure with a long transmembrane helix that via a hinge is connected to a surface-exposed amphiphatic helix that contacts loops of SecY. SecE of the archaeon *M. jannaschii* consist of only one TMS while the bacterial *Escherichia coli* SecE has three TMS [20]. The function of the two additional N-terminal helices is still unknown as truncation of these two helices in the *E. coli* SecE does not interfere with functionality although this truncation renders the complex less stable [21]. In a cryo-electron microscopy structure of the ribosome bound SecYEG complex reconstituted in lipid nanodiscs, the two additional TMS of SecE are peripherally localized from the complex contacting the ribosome [22]. The third subunit SecG is peripherally bound to SecY. This subunit is not essential for cell viability [23,24] but appears to stabilize the resting channel [25] whereby the loop region that connects the two TMS of SecG folds back into the translocation channel at the *cis*-side of the membrane [26].

SecYEG channel opening

The lateral gate opening may fulfil multiple functions. It creates a pathway for the insertion of transmembrane domains of integral proteins into the phospholipid bilayer [27], and provides a binding site for the incoming signal sequence of a translocating preprotein [28,29]. Signal sequences can be crosslinked to TMS2 and TMS7 of the Sec61p, the yeast SecY homolog in the endoplasmic reticulum [30]. The signal sequence intercalates into the lateral gate causes a conformational change involving TMS 7 and 10 and the plug domain. The movement of TMS7 towards TMS10 activates the channel and results in an opening of the central channel [29]. Three of the six pore ring residues are located on TMS2 and TMS7 and thus intercalation of the signal sequence between these TMSs can be directly coupled to channel opening [29]. SecE presumably stabilizes the two halves of SecY when the channel opens and the plug domain is displaced from its central position [14,19]. Channel opening is also influenced by SecA as evidenced by the structure of the *Thermotoga maritima* SecA-SecYEG complex (Fig. 2B) [15]. In this structure, the C-terminal halve of SecY has moved outwards resulting in a destabilization of the pore ring and a movement of the plug towards TMS7 but in this structure the pore has not yet fully opened. The lateral gate widens to approximately 5 Å providing a gap that may allow the signal sequence to insert and to sample the phospholipid bilayer. However, for translocation, the lateral gate needs to open allow a gap of about 10-12 Å between TMS2 and TMS7 [28][31].

The structure of *Geobacillus thermodenitrificans* SecYE-SecA complex with a covalently linked signal sequence in the translocation channel shows that the lateral gate undergoes large conformational changes [16]. Compared to the position of TMS7 in the closed channel state of the *M. jannaschii* SecY, this helix in the *G. thermodenitrificans* SecY has significantly changed position. The plug is shifted to the back of the channel and now is in close proximity to the TMS of SecE in line with crosslinking studies [19,32]. Compared to the *T. maritima* SecA-SecYEG structure, TMS7 of the *G. thermodenitrificans* SecY is tilted 10° relatively to the membrane and the periplasmic ends of TMS3 and TMS7 are now in close proximity. This change generates a large opening in the lateral gate that allows for signal sequence intercalation [16]. Interestingly, the plug of *G. thermodenitrificans* SecY is in β-strands structure which is different from the previously α-helical structures [14,33,34]. However, the amino acids residues in the plug domain are poorly conserved [14], while plug domain deletion in yeast Sec61p and *E. coli* SecY does not to interfere with cell viability but is only important for efficient translocation [35,36]. The plug domain is important for signal sequence recognition [35,37,38] as also suggested by molecular dynamics simulations which indicate that the plug domain samples the hydrophobicity of the incoming polypeptide region [39]. When the incoming polypeptide region is

highly hydrophobic, plug displacement does not occur and the polypeptide segment is directed to the lateral gate for membrane insertion. Crosslinking studies suggests a very large movement of the plug around 20-27 Å to the C-terminal loop of SecE to create an unobstructed path for the polypeptide to cross the channel [19,32]. However, *in vitro* translocation is not impaired when the plug is immobilized inside the channel [40], indicating that this large displacement is not critical for translocation. The plug domain role might mostly serve to stabilize the closed state of SecY [35], and to act as a periplasmic seal to prevents undesired ion leakage under those conditions.

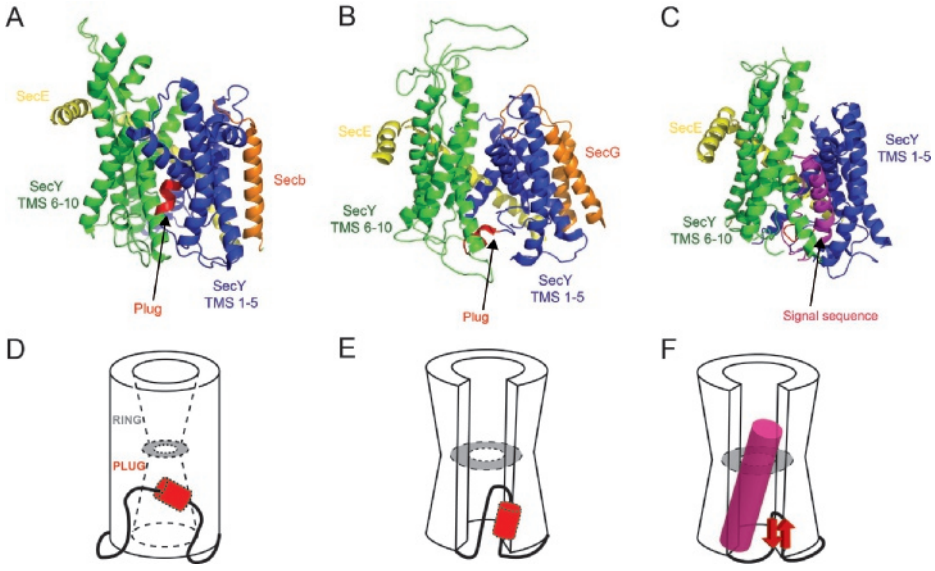

Fig. 2 Structural stages of the translocation channel. (A-C) The SecYEG/β crystal structures viewed from the membrane**.** SecY TMS 1-5 (blue), TMS 6-10 (green), plug domain (red), SecE (yellow) and SecG/β (orange) (D-F) cartoon illustration of SecYEG/β. The illustrations depict the opening of the constriction and movement of the plug domain depending on the state of the translocon. (A and D) *Methanococcus jannaschii* SecYEβ (PDB entry 1RH5), known as the closed or resting conformation. (B and E) *Thermotoga maritima* SecYEG co-crystalized with SecA (not shown) in a Mg-ADP-BeFx-bound transition state (PDB entry 3DIN) as a pre-open conformation. (C and F) *Geobacillus thermodenitrificans* SecYEG co-crystalized with SecA (not shown) and a signal sequence (magenta) latched into the lateral gate (PDB entry 5EUL), resembling an actively engaged translocation channel.

SecYEG pore constriction and width

A series of mutants have been identified in *sec* genes that allow the translocation of preproteins with a defective signal sequence or even when the signal sequence is completely absent. The protein localization (Prl) mutations impair the translocation proofreading activity and do not directly restore signal sequence recognition [41–44].

Prl mutation have been identified in *secY* (prlA), *secE* (prlG), *secA* (prlD) and *secG* (prlH). The most dominant *prl* variants are found in SecY [45–47]. These appear to destabilize the closed state of the translocation channel [25,37,45]. PrlA4 is the most studied *prl* mutant and its analysis has yielded major insights in the mechanism of suppression signal sequence [48]. PrlA4 carrying two mutations, F286Y in TMS7 and I408N in TMS10 with the localization at the facing side of TMS7 and the plug domain (TMS2a). The I408N mutation is responsible for the suppressor activity [49], while the F286Y mutation reduces the strength of I408N mutation. The open conformation of SecYEG is stabilized by the signal sequence, SecA or the ribosome and involves multiple interactions [14]. In contrast, the PrlA4 mutation alters the channel conformation. PrlA4 shows an increased binding affinity for SecA, an elevated SecA ATPase activity and the SecYE complex is destabilized [50,51]. Importantly, PrlA4 allows a more efficient translocation of native preproteins and to a lesser extent also supports translocation of preproteins with a defective signal sequence [49,52]. In this mutant, translocation is less dependence on the PMF [53]. This can be interpreted as a reduced proofreading activity, as signal sequence recognition is less stringent in PrlA mutants likely because the channel is already in a more open state.

Many of the PrlA mutants cluster around the pore constriction, and indeed electrophysiological studies indicate that these mutations cause an increased ion-leakage although the channel is rather resilient against individual mutations [54]. The hydrophobic constriction ring appears to function as a gasket to seal the translocation channel when a polypeptide crosses through the pore [14,18] preventing further ion leakage during translocation. The pore exhibits a high plasticity as it can be widened such that it supports the translocation of polypeptides with an internal disulfide bridge or a fold induced by a chemical crosslinker [55,56], although this translocation is highly PMF-dependent. SecY also supports the translocation of preproteins derivatized with a bulky fluorophores at the C-terminus [57]. A molecular dynamics simulation suggests that the pore can accommodate substrates with a cross-section of up to ~16 Å, without requiring opening of the lateral gate [17,58]. An experimental study demonstrates that substrate up to ~22 A [59] can pass the pore in a process that likely involves the opened lateral gate as a pore extension.

Oligomeric state of SecYEG

The oligomeric state of the SecYEG translocon has been a topic of long debate and controversy. SecYEG can be purified as a monomer [14,15] but depending on the detergent concentration also dimers and higher oligomers can be observed [60–62]. Crystallography and crosslinking experiments have suggested that SecYEG may form

dimers that are organized in either a 'back to back' [60,61] or 'front to front' arrangement [62]. In the 'back to back' arrangement, the dimer interface is formed by the SecE proteins of both protomers [63]. A cryo-EM study of the *E. coli* SecYEG bound to a ribosome-nascent polypeptide complex suggested a 'front to front' arrangement but only one of the channels carried a translocating polypeptide [62]. It was suggested that the lateral opening may coheres to generate a larger pore [59,64]. However, such an arrangement would hinder the interaction of SecY with accessories Sec proteins which interact with the lateral gate [65]. Although, SecYEG may aggregate into a dimeric structure, the functional role of the dimer has remained obscure. A crosslinking study showed that only one channel is used to translocate the polypeptide chain [66,60]. By the use of single SecYEG complexes reconstituted into nanodiscs as well as single molecule FRET studies, a single copy of SecYEG complex was shown to be sufficient for SecA binding and translocation, as well as for RNC binding [66–68]. This is in agreement with recent cryo electronmicroscopic studies on the structure of SecYEG bound to a RNC [15,16], and thus ample evidence supports the monomeric SecYEG complex as the minimal functional unit.

SECA, AN ATP-DEPENDENT MOTOR PROTEIN

SecA is a molecular motor that drives protein translocation by the conversion of chemical energy in the form of ATP into the movement of the polypeptide chain across the membrane [69]. As a soluble membrane peripheral protein, SecA associates with the membrane channel SecYEG but it also binds on its own to the phospholipid bilayer and to ribosomes. How SecA exactly drives protein translocation is still unknown. The structure of a soluble form SecA has been resolved in various states and from different species, as well as SecA-SecYEG co-structures, providing a glimpse on how function relates to structure.

SecA structure

SecA is a relatively large protein with a subunit mass of about 102 kDa. The protein can be divided into functional and structural subdomains (Fig. 3). The nucleotide binding domain (NBD) consists of NBD1 and NBD2 (also known as the intra-molecular regulator of ATPase 2; IRA2) which are essential for ATP binding and hydrolysis and for the translocation process [70]. The ATPase activity of SecA takes place at the interface of the two nucleotide-binding domains [71]. These NBDs form the so-called DEAD (Asp-Glu-Ala-Asp) motor which is also found in DNA/RNA helicases and contains the highly conserved Walker A and less-conserved Walker B motifs [72].

SecA recognizes preprotein substrates via its preprotein cross-linking domain, PPXD [73]. The PPXD is thought to have a dynamic role in the activation of the ATPase activity of SecA that is modulated by preprotein binding [74]. The carboxy domain (C-domain) of SecA can be divided into four subdomains: the α-helical scaffold domain (HSD) which interconnects all other domains of SecA [75], the α-helical wing domain (HWD), the intramolecular regulator of ATP hydrolysis 1 (IRA1) or two helix finger (2HF) which has been proposed to contact the preprotein during translocation [76], and the C-terminal linker domain (CTL). In *E. coli*, the CTL harbors a zinc finger which plays role in interaction with SecB [6,8] and phospholipids [77].

SecA exhibits a low basal ATPase activity [78]. The activity is stimulated in allosteric way by the binding of SecA to the molecular chaperone SecB, SecYEG, preprotein and anionic phospholipids [50,79–81]. The ATPase activity of cytosolic SecA is inhibited by IRA1, or the 2HF which forms a helix-loop-helix structure of the HSD that contacts both NBD2 and PPXD. SecA mutants with a defect in the IRA1/NBD2 interaction show an increased basal ATPase activity and are thus largely uncoupled from preprotein binding in their ATPase activity [82].

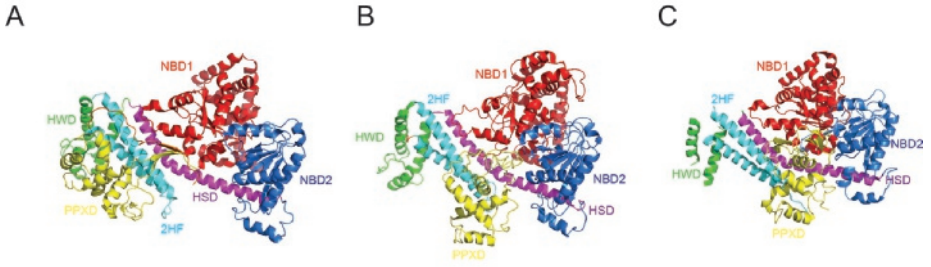

Fig. 3. Conformational states of SecA. Structures of (A) SecA from *Bacillus subtilis* (PDB entry 1M6N), (B) Mg-ADP-BeFx-bound SecA co-crystalized with SecYEG (not shown) from *T. maritima* (PDB 3DIN), and (C) Mg-ADP-BeFx bound SecA from *B. subtilis* engaged with the *G. thermodenitrificans* SecYEG and a signal sequence (not shown) (PDB entry 5EUL). The locations of the PPXD (yellow), NBD1 (red), NBD2 (blue), HWD (green), HSD (purple) and 2HF (cyan) are indicated. A large movement of the PPXD (yellow) suggests a closed (A) and open (B,C) conformation of SecA.

Oligomeric state of SecA

The functional oligomeric state of SecA has been a main element of controversy. Importantly, when SecA is purified from cells, it is mainly dimeric [83]. A multitude of studies demonstrate that SecA functions as a dimer [84–87]. In solution, SecA is found as an antiparallel homodimer [71,75]. SecA from *Thermus thermophilus* has been crystallized as a parallel dimer [88]. The SecA monomer-dimer equilibrium is affected by the ligands of SecA. SecA has been reported to be dimeric in the presence of synthetic

signal peptides [89] or when bound to phospholipid in the presence of nucleotides [90]. Convergingly, other studies suggests the opposite, that SecA monomerization occurs in the presence of phospholipids [89] and synthetic signal peptides [91]. It has been suggested that electrostatic and hydrophobic interactions maintain the SecA dimer, as the SecA monomer-dimer equilibrium is sensitive to high ionic strength and low temperature [85,92]. On the other hand, it is also known that SecA is highly thermolabile in the presence of phospholipids and rapidly loses its activity. However, this effect is counteracted by preprotein, and that activity is termed SecA lipid ATPase [93]. Since most of the studies on soluble SecA do not include the functional SecYEG bound state, the significance of the often conflicting observations on the quaternary state of SecA in solution in relation to the actual translocation mechanism remains obscure.

Other studies addressed the oligomeric state of SecA while bound to SecYEG. Single molecule analysis demonstrate that SecA remains dimeric during translocation [87,94] and is active as dimer [85,86,94,95]. Mutation induced monomerization severely reduces the SecA activity [96], but this defect can be overcome by non-physiologically high concentrations of SecA that restores the dimeric state [85,97]. The molecular chaperone SecB interacts with the SecYEG bound dimeric SecA and in this process the two positively charged C-termini of both SecA protomers bind to the opposing anionic flat surfaces of the SecB homotetramer [98]. A single molecule study showed that the dimeric SecA binds the SecYEG with high affinity, where one of the protomers binds tighly to SecYEG and the other protomer is bound to the SecYEG-bound SecA [85]. Additionally, a recent *in vivo* study demonstrated that SecA functions as a discrete anti-parallel dimer to drive protein translocation [99]. Although a structure of the SecA-SecYEG complex with a SecA monomer was solved [15,16], the high salt used for the crystallization likely induces the dissociation of the SecA dimer.

Binding partners of SecA

The N-terminal signal sequence of preproteins functions as a targeting signal [100,101]. Signal sequences have a conserved tripartite structure that consists of a hydrophobic α-helical region (H-domain) that is flanked by an N- and C-domain [102]. The C-domain contains hydrophilic residues and is usually devoid of positively charged residues. This region contains the signal peptidase cleavage site that meets the -1,-3 rule where these two positions contain amino acids with a small side chain [100,103]. The N-domain contains positively charged amino acid residues that help to orient the signal sequence in response to the transmembrane electrical potential, when the charged residues remain at the *cis*-side of the membrane. The length of the signal sequence varies, however the minimum length of *E. coli* signal sequence is 15-16 amino acids, with median of 22

amino acids [104]. Other studies suggest that the targeting information is contained in the mature region of a preprotein, and that the signal sequence only serves to initiate translocation [105,106]

Prior the translocation, the preprotein is maintained in an unfolded conformation, a state that is preserved by the molecular chaperone SecB or an attribute of the mature domain in the physicochemical surroundings of the cytosol. The SecYEG bound SecA directly interacts with SecB through its C-terminal zinc binding domain, and this interaction results in a transfer of the unfolded preprotein from SecB to SecA [6]. SecB exists as a homotetramer which is arranged as a dimer of dimer [107]. The tetramer contains two grooves of approximately 70 Å long, which run along either side of the tetramer and that are thought to be the peptide binding domains. A polypeptide may bind at both grooves and then likely is wrapped around the SecB complex [108,109]. In this respect, SecB binds preproteins in their folding core thus preventing folding when the protein emerges from the ribosome [110]. During the ATP-dependent initiation of translocation, SecB is released into the cytosol to bind another preprotein.

The interaction of SecA with phospholipids plays an important role in translocation as SecA binds to SecYEG via a phospholipid-bound intermediate state [93,111,112]. SecA binds to phospholipids through ionic interactions that involves the amphiphatic positively charged N-terminus [93]. Deletion of the N-terminal 20 amino acids of SecA results in a complete inactivation of SecA [93], but the activity can be restored by replacing this region by an artificial membrane tether [111]. However, membrane tethering is not sufficient, as insertion of a flexible linker between the N-terminus and the remainder of the SecA protein does not restore translocation [93]. The anionic phospholipid interaction of the N-terminus and its insertion into the membrane serves to enforce a conformational change onto SecA whereupon it is primed for high affinity SecYEG binding and ATP binding. The phospholipid bound catalytic intermediate of SecA likely also serves as a membrane que of SecA-preprotein complexes before SecA delivers the preprotein to SecYEG for translocation.

Structural mechanisms of SecA function

SecA undergoes a multitude of conformation changes during translocation [15], and various movements of the SecA motor domain have been suggested to occur upon ATP binding and ADP release [71,113]. In the *T. maritima* SecA co-crystal structure with SecYEG, where SecA is in transition state, i.e., an ADP beryllium fluoride bound state, the PPXD moves towards the NBD2 and away from the HWD [15,114] while the NBDs are in close proximity with the PPXD (Fig. 3B). Thus, this structure is considered as the

open conformation of SecA. Compared to the closed conformation of SecA derived from the crystal structure of the *Bacillus subtilis* SecA (PDB entry 1M6N) (Fig. 3A), the PPXD is located near the HWD [71]. The nucleotide-binding pocket between NBD1 and NBD2 is formed allowing the preprotein to bind in a groove between the NBD2 and PPXD. This opening stabilizes the preprotein-SecA interaction and increasing the rate of nucleotide exchange resulting in an activation of the ATPase activity of SecA [115,116]. The structure of the *G. thermodenitrificans* SecYE engaged with *B. subtilis* SecA [16] shows that SecA does not undergo further dramatic conformational changes as compared to *T. maritima* SecA-SecYEG structure (Fig. 3C). It has been proposed that SecA in its ATP bound state prevents the two halves of SecY from further movements that would drive these two halves apart.

The 2HF of SecA is inserted into the cytoplasmic opening of the SecY channel (Fig. 4) where the loop of the 2HF is in close proximity to the translocating preprotein at the entrance of the SecYEG pore [15]. The 2HF makes contact with the cytosolic loop 4 of SecY, and the insertion results in the opening of lateral gate by a rigid body movement of TMS 6–10 relative to TMS 1–5 [31]. The tip of the loop of the 2HF contains a highly conserved tyrosine residue which is crucial for translocation and can be replaced only by other bulky hydrophobic amino acids [76]. This has led to the proposition that the 2HF interacts with the unfolded polypeptide chain through hydrophobic interaction with side chains, although this model does not explain that SecA can mediate the translocation of preproteins with large stretches of glycine residues [117] which would only allow main chain interactions. Alternatively, the 2HF acts by opening the translocation channel through its interaction with the C4 loop of SecY. In this respect, chemical crosslinking of the 2HF with these loop regions did not interfere with translocation [118], suggesting the 2HF does not function as an ATP-dependent lever to push preproteins through the translocation channel but rather serves to push the two halves of SecY apart. Additionally, it has been proposed that the 2HF of SecA may act as a template by inserting the hairpin formed by the signal peptide and the early mature region of the preprotein [119].

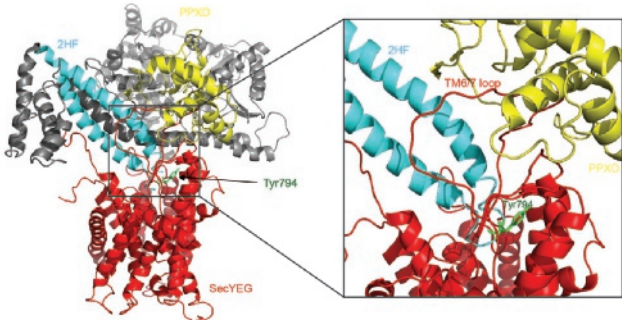

Fig. 4 Structure of *T.maritima* SecA-SecYEG complex. SecA penetrates into the SecYEG channel (red) via the so-called 'two helix finger' (2HF, light blue). The SecA PPXD (yellow) also binds to TM6/7 loop of SecYEG. The conserved tyrosine 794 is depicted in green.

TRANSLOCATION MODELS

In the recent years, major progress has been made in the structural elucidation of the proteins involved in protein translocation, and the possible mechanism of channel opening. However, the exact mechanism by which SecA drives translocation remains to be elucidated. In this section, we will discuss various models for the SecA mediated translocation mechanism.

Power stroke model

A large class of ATPases contain a RecA-like structural domain and use the energy of nucleotide binding and hydrolysis to move polypeptides or nucleic acids [72]. SecA has a DEAD box typically found in helicase, and thus a DNA helicase molecular mechanism [120] has been proposed that could potentially also be applied to SecA. In this so called power stoke mechanism, SecA is suggested to act as mechanical pump that pushes preprotein into SecYEG pore [81]. Herein, the coupled conformational changes of SecA induced by ATP binding and hydrolysis generate a mechanical force that drives translocation. As discussed in the previous section, the 2HF may function as an ATP-dependent lever that would support such power stroke mechanism and result in stepwise translocation (Fig. 5A). To applied the DNA helicase principle to SecA, SecA is required to multimerize in order to have multiple substrate binding site,s since monomeric SecA appears to have only one substrate binding [121,122]. One SecA protomer could act as the clamp and move the segment of the preprotein while the other SecA protomer traps the polypeptide chain in the channel since SecYEG is not able to make a stable anchor for preproteins [123]. In this model, the preprotein needs to be prevented from backsliding in the channel once SecA resets to its pre-translocation mode. Thus, a high cooperativity

is needed between the two protomers of SecA so that the preprotein is bound to one of the protomers at any given time.

Brownian ratchet model

In the Brownian ratchet model (Fig. 5B), SecA acts as the regulator for channel opening of SecYEG [124], while translocation occurs by Brownian movement of the unfolded preprotein through the channel. In the co-structure SecA-SecYEG, the 2HF is in contact with the C4-loop of SecY that connects TMS 6 and 7 [15]. Thus, movement of the 2HF could potentially result in an opening of the channel. Backsliding of the preprotein would be prevented by the SecA association and this may assure that the diffusion will only occur in only one direction, possibly further facilitated by folding of the polypeptide at the *cis* side of the membrane and/or by associations with the periplasmic domains of the SecDF complex [125]. This model explains the promiscuity of the system for polypeptide composition [126] but does not explain step-size translocation [9,81,123].

Push and Slide model

Another model of SecA-mediated protein translocation is the so called 'push-and-slide' mechanism [111] (Fig. 5C). This model combines the power stroke and Brownian Ratchet models, and explains earlier observations that SecA mediated translocation occurs step-wise, whereas in the absence of SecA association, the preprotein may slide within the translocation channel [123]. Mechanistically, the 2HF has been proposed to induce a power stroke by interacting with the preprotein and pushing it through the SecYEG pore [76,111]. Once ATP is hydrolyzed, the 2HF would return to its pre-translocation position and dissociate from the preprotein to allows passive sliding of the polypeptide chain into the channel. This model, however, does not explain that crosslinking of the SecA 2HF to the interacting loops on SecY does not interfere with translocation [118]. It is also not clear how the conserved tyrosine at the tip of the 2HF would release the polypeptide after ATP hydrolysis and how it would prevents backsliding in the SecYEG channel. Alternatively, step-wise translocation may arise from binding and release of SecA to and from the SecYEG channel as suggested by biochemical studies [123].

Reciprocating piston model

As mentioned at the previous section, SecA exists a dimer during translocation [86,87,94,127] but also monomeric states have been reported [15,96,127,128]. The reciprocating piston model combines the power stroke model with the SecA monomer-dimer transition [31] (Fig. 5D). Translocation is initiated by the binding of the dimeric SecA to SecYEG. Next, ATP hydrolysis induces SecA monomerization where one of the SecA monomers remains anchored to SecYEG to prevents backsliding of the partially

translocated preprotein, whereas the other monomer dissociates into the cytosol or to the membrane. Next, rebinding of another SecA monomer to SecYEG-SecA-preprotein complex promotes ATP independent translocation of a preprotein segment, while subsequent binding of ATP drives the translocation according to a power stroke mechanism. These steps are repeated until the preprotein is fully translocated though the channel. This model explains the two translocation stages in the process that were observed biochemically, *i.e*, translocation induced by SecA binding to the preprotein, and an ATP-dependent translocation step [81,123]. Furthermore, complete dissociation of SecA from SecYEG may allow translocation by Brownian diffusion and permit PMF-driven translocation as will be discussed in the following section.

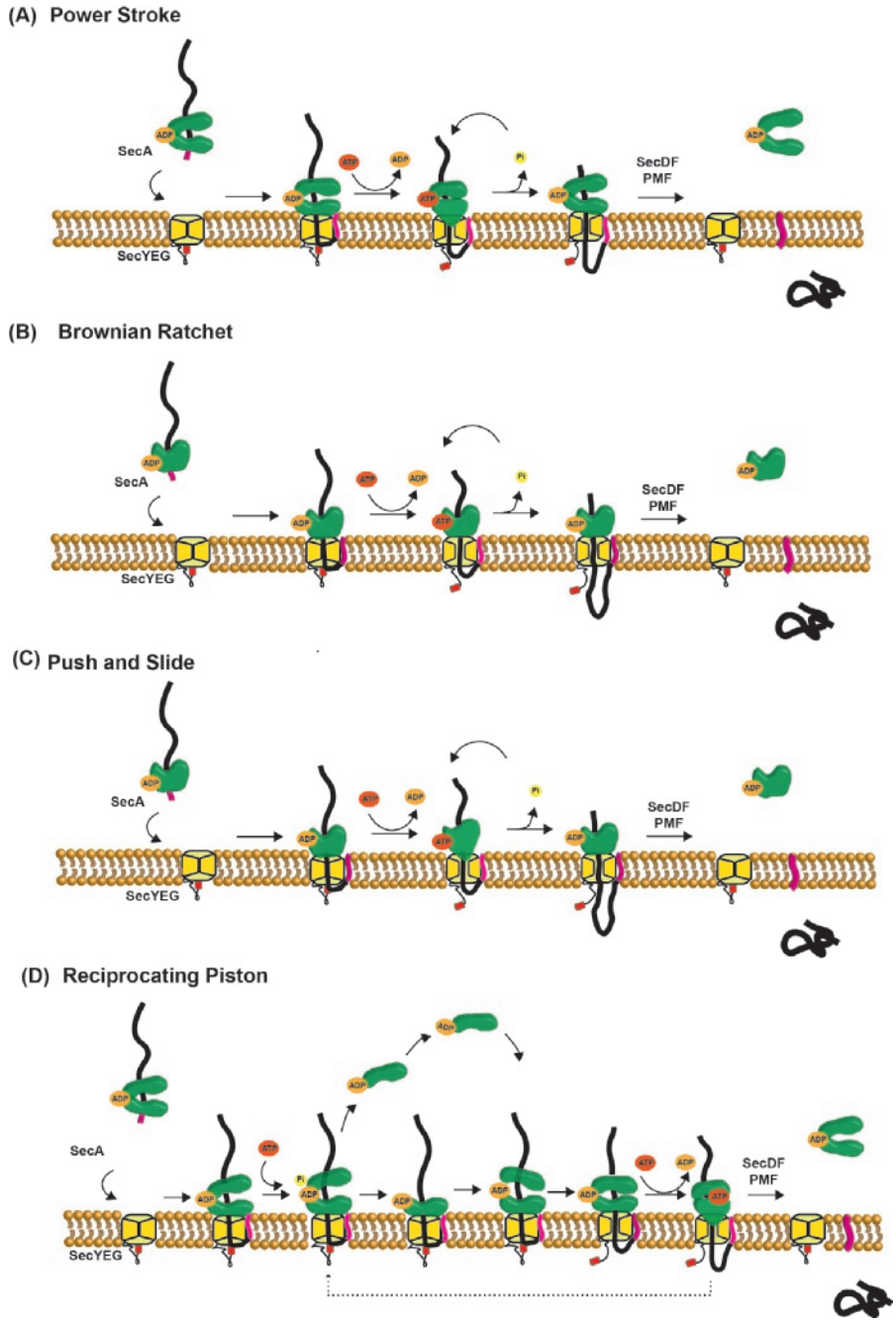

Fig. 5. Proposed models of SecA-mediated protein translocation. (A) Power stroke: ATP binding and hydrolysis induces conformational changes of SecA that result in a mechanical force on the preprotein pushing it through the SecYEG channel. In this model, oligomerization of SecA

is required to prevent backsliding of the preprotein. (**B) Brownian ratchet**: SecA regulates the SecYEG channel opening via the 2HF of SecA and allowing the protein translocation via diffusion. Trapping and release of the translocating preprotein at the cis-side results in translocation, while SecA may fulfil an additional function by opening the translocation channel. The oligomeric state of SecA is not explicitly shown in this model. **(C) Push and slide**: This model uses both SecA dependent-pushing and Brownian motion. The oligomeric state of SecA is not explicitly shown in this model. **(D) Reciprocating piston:** This model is a combination of a power stroke mechanism with the conversion of dimeric-monomeric SecA. Repeated cycles of SecA monomerization-rebinding and ATP binding-hydrolysis yields in a step-wise translocation process. In none of the above models, the exact role of the PMF and SecDF is included which contribute to efficient translocation.

ROLE OF THE SECDF COMPLEX

Importantly, the aforementioned models do not take the role of the PMF into account. Although SecA can drive translocation *in vitro* on its own, protein translocation *in vivo* is strongly dependent on the PMF. *In vivo*, SecA may mainly serve to initiate translocation by releasing the signal sequence domain and the early mature domain of a preprotein as a looped structure into the SecY pore then allow for a directed diffusional or power stroke translocation mechanisms that is further accelerated by the PMF and the SecDF complex [12]. Indeed, *in vitro* preprotein translocation at low SecA concentrations is highly PMF dependent while in the presence of high concentrations of SecA, ATP-driven translocation dominates [129,130]

SecDF is a subcomplex that associates with the SecYEG translocon to form the holo-translocon complex [125]. The complex exists as two individual proteins SecD and SecF or as a large SecDF fusion protein. In the crystal structure of SecDF from *Thermus thermophilus and Deinococcus radiodurans*, SecDF exists as a monomer with 12 TMS, 6 TMS each in both SecD and SecF. The protein (complex) also contains 6 periplasmic domain (P1-P6) where P1 and P4 form a periplasmic protruding structure [131,132]. P1 has been proposed to interact with the polypeptide substrate, and a conformational change or movement of P1 may results in a PMF dependent pulling action by SecDF at the periplasmic side of the membrane [132,133]. Indeed, the late stages of protein translocation process can occur without ATP and are SecDF and PMF dependent [134]. In this process, large unfolded regions of the preprotein can be translocated.

CONCLUDING REMARKS

A multitude of biochemical and biophysical studies has been made to understand the molecular mechanism of SecA in protein translocation. The X-ray structural data of different states of the SecA protein both in the absence and presence of SecYEG association has revealed various conformations providing further insight into structural

basis of protein translocation. However, still many of the mechanistic questions on SecA mediated protein translocation have remained unresolved, and although translocation exhibits features typical observed for a power stroke and Brownian diffusion mechanisms, it remains unclear how this process is directed by the SecA protein. Also, the exact role of the SecA dimer remains to be resolved. To unify potentially conflicting results, the process needs to be examined at the single molecule level to reveal the dynamic interplay between the components and identify their role at the different stages of the process. Also, current mechanistic insights should be integrated and combined with *in vivo* studies on protein translocation, in order to understand how this process is coordinated within the crowded intracellular environment of the cytosol and cytoplasmic membrane. Considering the dual role of the translocon in protein translocation and membrane protein integration, a further unresolved questions is whether translocons exist in the membrane with a specialized function or whether the translocon composition is highly dynamics and assembled on demand.

SCOPE OF THIS THESIS

This study aims to decipher the molecular mechanism of SecA-mediated protein translocation using combined biochemical and enzymatic approaches.

Chapter 1 provides a review on the current understanding of the structure and function of the Sec translocon, with the focus on the protein conducting channel SecYEG and the motor protein SecA.

Chapter 2 investigates the role of the 2HF of SecA in protein translocation. Here, we analyzed the importance of the 2HF in protein translocation by extending and truncating the length of the 2HF. *In vivo* complementation assay were employed to analyze the activity of the SecA 2HF mutants in cell viability. Furthermore, SecA 2HF mutants were tested *in vitro* with translocation and SecYEG binding assays. Our data demonstrate a complete functionality of a SecA variant with a symmetrically shorted 2HF but suggests that the truncation results in an increased energetic requirement for translocation.

Chapter 3 analyzes the tolerance of SecYEG for the preproteins that were chemically modified with non-polypeptide constituents. A fluorophore and polymeric hydrophilic oligoethylene glycol chains were introduced into the mature region of proOmpA. Our data demonstrates that the translocon is remarkably promiscuous for its polypeptide substrates and accepts such chemical modifications except present in at the extreme mature N-terminus of the preprotein where it possibly interferes with initiation of translocation.

Chapter 4 presents a study to follow the interaction between the 2HF of SecA with SecYEG using a FRET based approach. Herein, a set of monocysteine SecA and SecY mutants were generated and labeled with a donor and an acceptor fluorophore, respectively. FRET was used to examine possible dynamic changes in the interaction but once the interaction was established, no translocation dependent FRET changes were observed suggesting the SecA 2HF interacts stably with SecYEG.

Finally, in **Chapter 5**, findings reported in this thesis are summarized with a future perspective on the studies on the mechanism of the SecA-mediated protein translocation.

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